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Th1 and Th2 cytokine production is suppressed at the level of transcriptional regulation in Kawasaki disease

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SUMMARY

To clarify the functional state of T cells in Kawasaki disease, we analysed mRNA expression levels of Th1/Th2 cytokines (IFN-γ and IL-4) along with Th1/Th2-inducing transcription factors, T-bet and GATA-3, which play pivotal roles in the development of Th1 and Th2 cells, respectively. By real-time PCR, IFN-γ mRNA levels in peripheral blood mononuclear cells (PBMNC) were significantly decreased in Kawasaki disease patients compared with those with measles, and tended to be lower than those in healthy controls. T-bet mRNA levels were significantly decreased in patients with Kawasaki disease compared with healthy controls. In addition, IL-4 and GATA-3 mRNA levels were significantly decreased in Kawasaki disease compared with healthy controls. Regulatory cytokine mRNA levels (TGF-β and IL-10) were also decreased in Kawasaki disease. The mRNA levels of IFN-γ showed a significant positive correlation with those of T-bet in Kawasaki disease. These results suggest that the suppressed function of Th1 and Th2, associated with the suppression of both T-bet and GATA-3 gene expression, may be one of the immunological characteristics of Kawasaki disease.

Keywords Kawasaki disease T-bet GATA-3 IFN-γ IL-4

INTRODUCTION

When T helper (Th) cells are activated, they mainly differentiate into two functionally distinct subsets, Th1 and Th2 cells. Transcription factors, T-bet and GATA-3 play important roles in the differentiation of Th1 and Th2 subset, respectively [1–4]**.** Th1 cells play an important role in cellular immunity by secreting IL-2 and IFN-γ, and Th2 cells have the helper function for the development of antibody producing B cells by secreting IL-4, IL-5, IL-6, and IL-10 [5,6].

Kawasaki disease is an acute illness of unknown aetiology characterized by prolonged fever, diffuse mucosal inflammation, oedema of hands and feet, skin rash and nonsuppurative lymphadenopathy [7]. Histopathological findings indicate panvasculitis with endothelial necrosis and infiltration of mononuclear cells (MNC) into small- and medium-sized blood vessels [7–9]. Although activation of immune systems and production of various cytokines have been reported, the role of T cells and the functional state of Th1 and Th2 cells in Kawasaki disease are still controversial [10–15]**.** Although IFN-γ, IL-4 and IL-10 levels are increased in Kawasaki disease [10–12], it was reported that IFN-γ production from T cells was decreased [13], and that there was no significant difference in the percentages of IL-4 producing T cells in the peripheral blood between patients with Kawasaki disease and healthy controls [14,15].

To clarify the functional state of T cells in Kawasaki disease, we analysed mRNA levels of T-bet and GATA-3 along with IFN-γ and IL-4 in peripheral blood mononuclear cells (PBMNC). We found that mRNA levels of T-bet and GATA-3, in addition to IL-4, were significantly decreased in Kawasaki disease compared with healthy controls, suggesting the suppressed state of both Th1 and Th2 function at the levels of transcriptional regulation. These results support that peripheral blood T cells are functionally suppressed in terms of Th1 and Th2 cytokine production, rather than activated during acute Kawasaki disease.

MATERIALS AND METHODS

Patients

Peripheral blood samples were obtained from 11 patients (age range 5 months – 8.5 years) who met diagnostic criteria for Kawasaki disease [16], age matched 12 healthy donors (age range 4 months – 6 years), and 9 patients with measles (age range 10–34

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months) diagnosed by serological examination, at Kyushu University Hospital, and Fukuoka Children's Hospital and Medical Centre for Infectious Diseases. All peripheral blood samples from patients with acute Kawasaki disease were collected before intravenous gammaglobulin therapy. The peripheral blood samples from measles were obtained from the patients diagnosed by the clinical manifestations. The diagnosis of all mealses patients was confirmed by serological examinations. All blood samples were drawn immediately after presentation to hospital. Heparinized cord blood samples were obtained from umbilical cord veins of neonates who had no hereditary disorders or haematological abnormalities, and were analysed within 24 h. All samples were collected after informed consent was obtained from patients or their parents. This study was performed according to the Regional Committee of Ethics for Human research at Faculty of Medicine of Kyushu University.

RNA extraction and reverse transcription

PBMNC were separated by Ficoll-Hypaque (Amersham Pharmacia-Biotech AB, Uppsara, Sweden) density gradient centrifugation. Total RNA was extracted and first-strand cDNA was synthesize by First-strand cDNA Synthesis Kit (Amersham Biosciences AB, Uppsara, Sweden).

Quantitative PCR

We quantified mRNA by TaqMan real-time PCR method (PE-Applied Biosystems, Tokyo, Japan). The intensities of the fluorescent dyes in each reaction were read automatically during PCR cycling in Sequence Detector 7700 (PE-Applied Biosystems). The real-time data were analysed with sequence detector software (version 1·6.3; Perkin Elmer, CA, USA). Each specimen was run in duplicate. PCR primers and Taq Man probes are shown in Table 1.

In vitro *differentiation of cord blood T cells into Th1 and Th2 cells*

Cord blood mononuclear cells were separated by Ficoll-Hypaque (Amersham Pharmacia-Biotech) density gradient centrifugation. $CD4^+$ T cells were purified by cell sorter (EPICS ALTRA, Immunotech, Miami, FL, USA), and cultured in RPMI1640 with IL-2 (100 U/ml) and PHA (100 ng/ml). For differentiation to Th1 cells, IL-12 was added (2·5 ng/ml), and for differentiation to Th2 cells, IL-4 (10 ng/ml) and anti-IL-12 (10 μg/ml) were added. After the stimulation for 1 and 3 days, total RNA was extracted, and cDNA was synthesized [17,18].

Statistical analysis

A difference in mRNA expression levels of PBMNC among patients with Kawasaki disease, measles, and healthy controls was analysed by Kraskul-Wallis test and multiple comparisons (Scheffe). The correlation of mRNA levels between IFN-γ and T-bet, or IL-4 and GATA-3 were analysed by Spearman's rank sum test.

RESULTS

We first analysed the changes of mRNA levels of Th1/Th2 cytokines (IFN-γ and IL-4) and Th1/Th2-inducing transcription factors (T-bet and GATA-3) during the course of differentiation into Th1 and Th2 cells from naïve $CD4^+$ T cells *in vitro* using cord blood [17,18]. IFN-γ and T-bet mRNA expression levels started to increase on day 1 and showed a maximal increase on day 3 after stimulation with IL-12 (Fig. 1a). Similarly, IL-4 and GATA-3 mRNA expression levels were maximal on day 3 after stimulation with IL-4 and anti-IL-12 (Fig. 1b). These results were consistent with those in mouse $CD4^+$ T cells after TCR/CD28 stimulation [19].

Then, we analysed IFN-γ and IL-4 mRNA levels of PBMNC in patients with active Kawasaki disease. IFN-γ mRNA levels in Kawasaki disease were significantly decreased compared with measles, and tended to be lower than those in healthy controls (Fig. 2a). IL-4 mRNA levels were significantly decreased in Kawasaki disease compared with healthy controls (Fig. 2b). Neither IFN-γ nor IL-4 mRNA levels in patients with measles were significantly different from those in healthy controls (Fig. 2). On the other hand, both T-bet and GATA-3 mRNA levels in Kawasaki disease were significantly lower than those in controls (Fig. 3). In measles, GATA-3, but not T-bet mRNA levels were lower than those in controls (Fig. 3). In purified $CD4^+$T cells, T-bet, Gata-3, IFN-γ, and IL-4 mRNA levels were decreased in acute Kawasaki desease compared with healthy controls, although only IL-4 mRNA expression levels did not reach the

Table 1. PCR primers and Taq Man probes used in this study

T-bet	
Tak Man probe	5′-(FAM)-CCC CTT TGC CCAA AGG ATT CCG G-(TAMRA)-3′
Forward primer	5′-GCC TAC AGA ATG CCG AGA TTA CT-3′
Reverse primer	5′-GGA TGC TGG TGT CAA CAG ATG-3′
GATA-3	
Tak Man probe	5′-(FAM)-AGA ACC GGC CCC TCA TTA AGC CCA-(TAMRA)-3′
Forward primer	5′-GCG GGC TCT ATC ACA AAA TGA-3′
Reverse primer	5′-GCT CTC CTG GCT GCA GAC AGC-3′
IFN-γ	
Tak Man probe	5′-(FAM)-CGG TAA CTG ACT TGA ATG TCC AAC GCAA-(TAMRA)-3′
Forward primer	5′-ACG AGA TGA CTT CGA AAA GCT G-3′
Reverse primer	5′-TTT AGC TGC TGG CGA CAG TTC-3′
IL-4	
Tak Man probe	5′-(FAM)-CCT GGC GGG CTT GAA TTC CTG TCC T-(TAMRA)-3′
Forward primer	5′-CAC AAG CAG CTG ATCC GAT T-3′
Reverse primer	5′-ACG TAC TCT GGT TGG CTT CCT T-3′

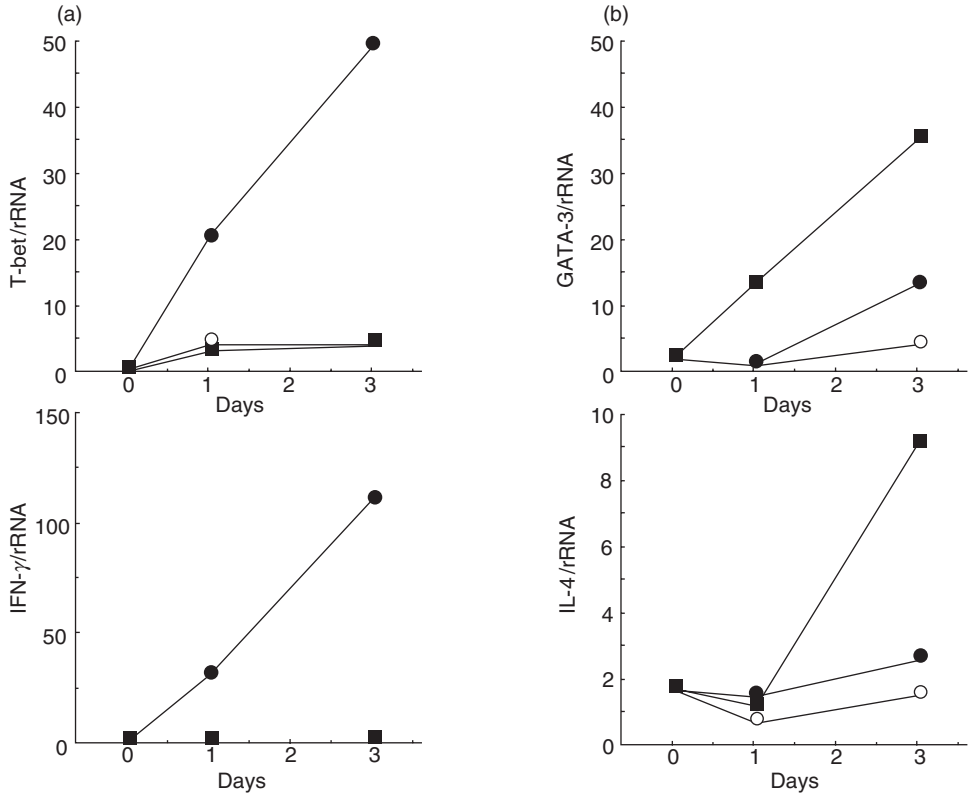

Fig. 1. Induction of T-bet and Gata-3 mRNA in cord blood MNC after culture toward Th1 or Th2 differentiation. Cord blood MNC were culture with PHA and IL-2, as described in materials and methods. Th1 and Th2 cells were induced in the presence of IL-12 (●), and IL-4 and anti-IL-12 (■), respectively; (○) medium alone. The mRNA levels of T-bet, GATA-3, IFN-γ, IL-4 were measured by real-time PCR method. The rRNA was used as internal control.

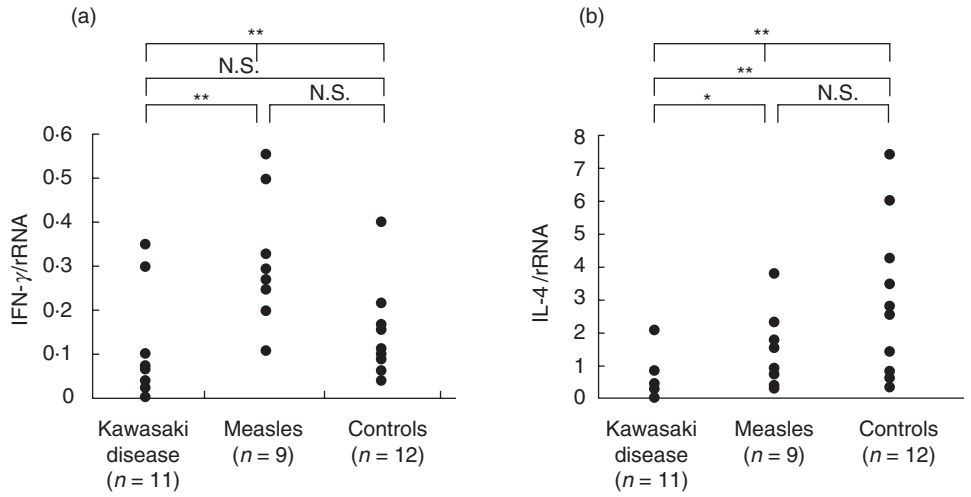

Fig. 2. IFN-γ and IL-4 mRNA levels in Kawasaki disease. The mRNA levels of (a) IFN-γ and (b) IL-4 in PBMNC of Kawasaki disease were analysed by real-time PCR and compared with those of measles and healthy controls. The rRNA was used as internal control. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$; NS, not significant.

statistical significance (data not shown). As shown in Fig. 4, regulatory cytokines (TGF-β and IL-10) mRNA levels were also decreased in acute Kawasaki disease.

To analyse the significance of T-bet and GATA-3 in the differentiation of Th1 and Th2 cells *in vivo*, we investigated the correlation of mRNA levels of T-bet and IFN-γ, or GATA-3 and IL-4 in PBMNC of Kawasaki disease. The mRNA levels of T-bet showed a significant positive correlation with those of IFN-γ in patients with Kawasaki disease (Fig. 5a), although GATA-3 and IL-4 mRNA levels had no significant correlation (Fig. 5b).

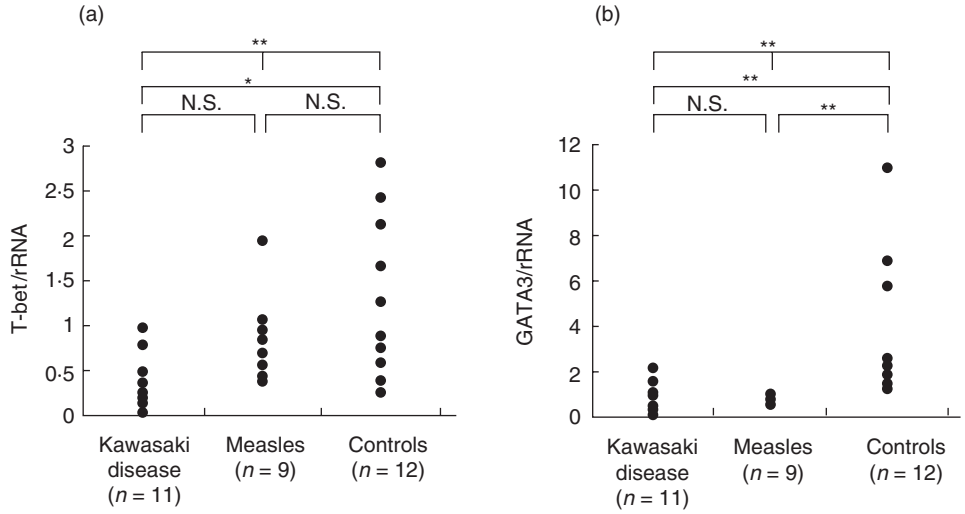

Fig. 3. T-bet and GATA-3 mRNA levels in Kawasaki disease. The mRNA levels of (a) T-bet and (b) GATA-3 in PBMNC of Kawasaki disease were analysed by real-time PCR and compared with those of measles and healthy controls. The rRNA was used as internal control. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$; NS, not significant.

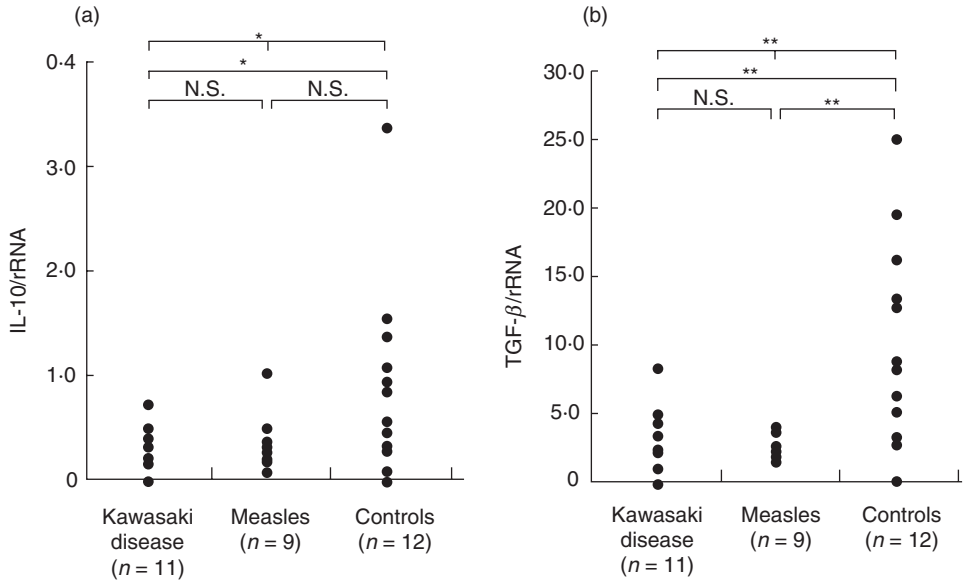

Fig. 4. IL-10 and TGF-β levels in Kawasaki disease The mRNA levels of (a) IL-10 and (b) TGF-β in PBMNC of Kawasaki disease were analysed by real-time PCR and compared with those of measles and healthy controls. The rRNA was used as internal control. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$; NS, not significant.

DISCUSSION

The functional state of T cells in Kawasaki disease has been still controversial. It has been reported that the numbers of activated T cells bearing HLA-DR were increased [20], and production of IL-2, TNF-α and IFN-γ was enhanced after stimulation with PHA in PBMNC of the patients with Kawasaki disease [21]. Serum levels of IFN-γ and IL-4, cytokines predominantly produced by T cells, were reported to be increased in Kawasaki disease compared with healthy controls [11,12]. In addition, NF-κB activation was observed in peripheral blood T cells [22]. On the contrary, Matsubara *et al*. [23] suggested that T cells in the peripheral blood of patients with Kawasaki disease were not activated because of the low expression levels of intracellular CTLA-4. In Kawasaki disease, increases of IFN-γ or IL-4 producing T cells were not observed by the stimulation *in vitro* [13], and by the analysis of intracellular cytokines using flow cytometory [14,15].

In this study, we focused on the mRNA levels of the Th1 and Th2 cytokines, and also of transcription factors which have important role in the differentiation of Th1 and Th2 cells, to obtain more direct information concerning the function and regulation of Th1/Th2 cells *in vivo* during acute Kawasaki disease. T-bet, a member of T-box family of transcription factors, is a master regulator of Th1 lineage commitment [4,24]. T-bet is induced by STAT-1-

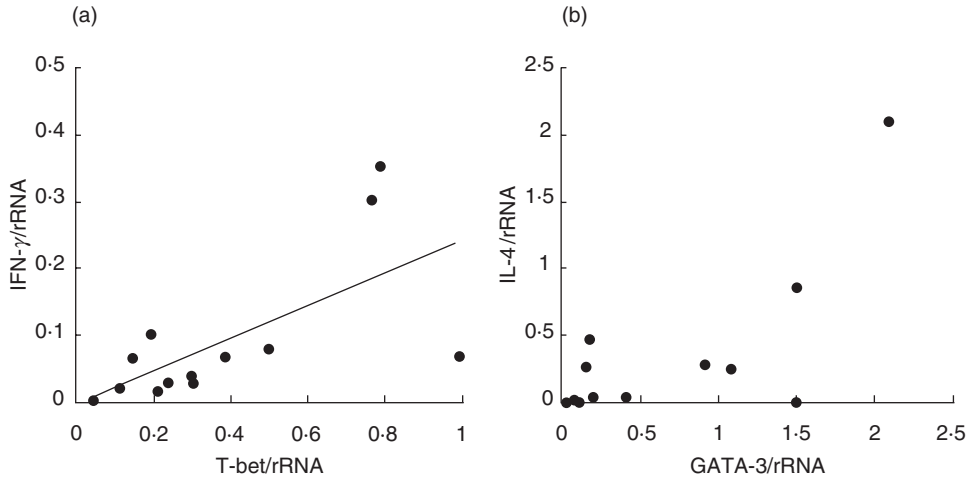

Fig. 5. Correlation of mRNA levels of IFN-γ and T-bet, or IL-4 and GATA-3 in PBMNC of Kawasaki disease. The correlation of mRNA levels between IFN-γ and T-bet, or IL-4 and GATA-3 were analysed by Spearman's rank sum test. The mRNA levels of IFN-γ and T-bet showed a significant positive correlation ($\rho = 0{\cdot}748$, $P < 0{\cdot}05$).

mediated signals and strongly promotes IFN-γ and IL-12 receptor β2 expression [25]. It is reported that T-bet expression correlates with IFN-γ gene expression and induces endogenous IFN-γ production [19]. On the other hand, GATA-3 is a zing-finger protein that is expressed during the course of Th2 differentiation in response to IL-4, and activates and stabilizes the expression of IL-4, IL-5, and IL-13 [3,19,26,27]. Actually, T-bet was up-regulated in active Crohn's and Behcet's diseases, in which Th1 cells are known to be involved in their pathophysiologies [28,29]. In contrast, GATA-3 mRNA expression was significantly increased in the airway inflammatory cells in asthmatic patients, predominantly in T cells, eosinophils, and mast cells. In addition, the density of cells expressing GATA-3 correlated with the numbers of cells expressing IL-5 mRNA, reduced airway caliber, and airway hyperresponsiveness [30]. This is the first report that demonstrated decreased mRNA levels of both T-bet and GATA-3 in acute Kawasaki disease, which might be one of its pathophysiological characteristics (Figs 2 and 3). It suggests that peripheral blood T cells are rather suppressed in the potential to produce IFN-γ or IL-4 at the levels of transcriptional regulation. In addition, T-bet mRNA levels showed significant correlation with IFN-γ (Fig. 5). These results suggest that T-bet and GATA-3 also play predominant roles in regulating IFN-γ and IL-4 producing potentials of T cells in Kawasaki disease. IL-10 and TGF-β did not seem to be associated with the decreased Th1 and Th2 functions (Fig. 4).

The present study can not exclude a possibility that T cells play a role in the pathophysiology of Kawasaki disease. A decrease of T cells with the potential of Th1 or Th2 cells in the peripheral blood during acute Kawasaki disease might be due to the infiltration of activated T cells into the inflammatory tissues. Actually, the lesions in skin biopsy specimens of a Kawasaki disease patient were infiltrated by HLA-DR$^+$ T cells [31]. Furukawa *et al*. [32] suggested that activated T cells with bright LFA-1 α and LFA-1 β were temporarily withdrawn from peripheral circulation during acute Kawasaki disease. Alternatively, an increased apoptosis of T cells might occur after activation during the course of Kawasaki disease. Kim *et al*. [33] reported that apoptosis of PBMNC was increased in Kawasaki disease compared with healthy and febrile controls.

Analyses of the T cell functions in tissues, and identification of the molecules which regulate the function of T-bet and GATA-3 would be necessary to further clarify a possible role of T cells in the pathophysiology of Kawasaki disease.

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